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# RAS and RAF-1 Form a Signalling Complex with MEK-1 but Not MEK-2

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**Recent studies have demonstrated the existence of a physical complex containing p21$^{ras}$ (RAS), p74$^{raf-1}$ (RAF-1), and MEK-1. Although it is clear that formation of this complex depends on the activation state of RAS, it is not known whether this complex is regulated by the activation state of the cell and whether MEK-2 is also present in the complex. To analyze the regulation and specificity of this complex, we utilized immobilized RAS to probe lysates of cultured NIH 3T3 fibroblasts and analyzed the proteins complexing with RAS following serum starvation or stimulation. Complex formation among RAS, RAF-1, and MEK-1 was dependent only on RAS:GMP-PNP and not on cell stimulation. Incubations of lysates with immobilized RAS depleted all RAF-1 from the lysate but bound only a small fraction of cytosolic MEK-1, and further MEK-1 could bind immobilized RAS only if exogenous RAF-1 was added to the lysate. This indicates that binding of MEK-1 to RAS depends on the presence of RAF-1 or an equivalent protein. In contrast to MEK-1, MEK-2 was not detected in the RAS signalling complex. A proline-rich region of MEK-1 containing a phosphorylation site appears to be essential for signalling complex formation. Consistent with the preferential binding of MEK-1 to RAS:RAF-1, the basal activity of MEK-1 in v-*ras*-transformed cells was found to be elevated sixfold, whereas MEK-2 was elevated only twofold, suggesting that the RAS signalling pathway favors MEK-1 activation.**

p21$^{ras}$ (RAS) is a membrane-associated guanine nucleotide-binding protein which is active when bound to GTP and inactive when bound to GDP (31). Activation of RAS occurs in response to numerous agonists associated with growth and differentiation, reflecting the importance of RAS as a molecular switch regulating diverse cellular responses (22). The ratio of GTP to GDP can be regulated either by controlling the rate of nucleotide exchange or by regulating the rate of GTP hydrolysis (10, 31). Activation of RAS is sufficient to stimulate a kinase cascade leading to the activation of mitogen-activated protein (MAP) kinases (40, 41); in most cases, activation of RAS is also necessary for activation of MAP kinases (9, 26, 36).

Recent studies have clarified some of the components which lie downstream from RAS in the regulation of MAP kinases. The MAP kinases are dually phosphorylated on threonine and tyrosine by MAP kinase kinases (MKKs or MEKs) (1, 2, 44), and at least two MEKs, MEK-1 and MEK-2, are known to be able to catalyze this phosphorylation in vitro (3, 7, 16, 27, 33, 42, 43, 46). MEK-1 is a phosphoprotein, regulated by serine/threonine phosphorylation, and a substantial body of evidence suggests that p74$^{raf-1}$ (RAF-1) is capable of catalyzing this activating phosphorylation (8, 14, 17, 21, 25, 29). Although v-RAF can phosphorylate MEK-2 in vitro (42), it is as yet unclear how MEK-2 is regulated in vivo and whether there are functional or regulatory differences between MEK-1 and MEK-2.

The role of RAS in the regulation of the MAP kinase cascade is thought to involve direct physical association between RAS and RAF-1 (11, 24, 37–39, 45), possibly by forming a ternary complex with MEK-1 (15, 24, 37). The role of the interaction between RAS and RAF-1 is thought to be to bring RAF-1 to the membrane (19, 35), but the mechanism of RAF-1 activation is at present unknown, as is the fate of the RAS:RAF-1 complex once RAF-1 activation occurs. Most studies on enzymatic activation of RAF-1 have focused on the role(s) of protein kinases, and it appears that phosphorylation by a variety of kinases can play a role in RAF-1 activation (13, 20).

Because of the probability that the ternary complex among RAS, RAF-1, and MEK plays an important role in signal transduction, we have characterized the specificity and regulation of the formation of this complex. We find that the RAS:RAF-1 MEK-1 interaction is reduced by a peptide corresponding to the effector domain of RAS, consistent with the genetic data which indicate that RAF-1 is an effector for RAS. We also find that RAF-1 from both quiescent cells and stimulated cells binds to RAS, suggesting that the RAS:RAF-1 interaction depends on the cycle of GTP binding and hydrolysis, and not on events downstream. Depletion experiments indicate that RAF-1 is able to mediate the binding of MEK-1 to RAS. Finally, we show that MEK-1 but not MEK-2 or mutant MEK-1 (T292A) binds to RAS:RAF-1 and that the basal activity of MEK-1 is elevated in v-*ras*-transformed cells relative to MEK-2 activity, suggesting that the Ras signalling pathway preferentially activates MAP kinase via MEK-1.

## MATERIALS AND METHODS

**Cell propagation and preparation of lysates.** NIH 3T3 cells, c-RAS-overexpressing and v-*ras*-transformed NIH 3T3 cells (a gift from D. Lowy), and v-*raf*-transformed NIH 3T3 cells (a gift from R. Jove) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, and CCL39 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% bovine calf serum and 5% fetal bovine serum. At >90% confluence, the cells were serum starved for 16 h and then stimulated with 20% fetal bovine serum for 5 min. Cells were quickly chilled and washed twice with ice-cold phosphate-buffered saline (PBS) and then were scraped into

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polypropylene tubes and spun to a pellet. Pellets were resuspended in p21 buffer (24) and homogenized with a hand-held blender. Cell debris was removed by centrifugation at 15,000 × *g* for 5 min, and membranes were pelleted by 100,000 × *g* centrifugation at 4°C for 45 min. Supernatant fractions contained essentially all of the cellular RAF-1 and MEK proteins and were used for studies with immobilized RAS.

For assays of MEK activity, cells were lysed in a solution of 50 mM Tris (pH 7.5), 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 40 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 200 μM $Na_3VO_4$, 1 mM benzamidine, 1 μM okadaic acid, 40 mM 4-nitrophenyl phosphate (PNPP), 1 μg of pepstatin A per ml, 1 μg of aprotinin per ml, and 1 μg of leupeptin per ml. Lysates were cleared of insoluble material by centrifugation at 15,000 × *g* for 15 min prior to immunoprecipitations.

**Cells expressing HA-tagged mutant MEK proteins.** MEK cDNAs were tagged with an epitope from influenza virus hemagglutinin (HA), and mutants constructed in this background will be described elsewhere (4). These constructs were used to transfect CCL39 hamster fibroblasts, and clones were established on the basis of resistance to G418. Expression was verified by immunoprecipitation with monoclonal anti-HA antibody 12CA5 and by Western blotting (immunoblotting).

**Assay of lysates with immobilized RAS.** Between 10 and 100 μg of lysate was incubated with immobilized RAS loaded with GDP or the GTP analog guanylyl-imidodiphosphate (GMP-PNP) for 30 min at 4°C with constant rotation. Samples were processed essentially as described elsewhere (24).

**Antibodies and proteins.** Rabbit anti-MEK-1 antiserum was raised against a peptide (CSTIGLNQPSTPTHAASI) and affinity purified with the same peptide. Anti-MEK-2 amino-terminal rabbit antiserum was provided by S. Pelech. Anti-HA monoclonal antibody 12CA5 was purchased from Berkeley Antibody Co. Affinity-purified anti-RAF-1 antiserum reactive against the carboxy-terminal peptide was provided by P. Dent and T. W. Sturgill. Recombinant baculovirus expressing RAF-1 was provided by R. Jove. Infected Sf9 cells were homogenized in p21 buffer as described above, and membranes were removed by centrifugation at 100,000 × *g*. RAF-1 made up approximately 5% of the total cytosolic protein.

**Assays of MEK activity.** Anti-MEK antisera or preimmune sera (20 μl per sample) were used to immunoprecipitate the appropriate MEK protein and washed twice in lysis buffer and then twice in reaction buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 10 mM magnesium acetate [MgOAc]). Immune complex kinase assays were performed in a solution of 50 mM HEPES (pH 7.4), 10 mM MgOAc, 1 mM dithiothreitol, 5 μM ATP, 20 μCi of [γ-$^{32}$P]ATP, and 2 μg of kinase inactive MAP kinase (K52R) per sample for 30 min at 30°C.

## RESULTS

**Association of cellular RAF-1 and MEK-1 with immobilized RAS.** Previous investigations with in vitro systems, as well as yeast expression studies, have shown that RAF-1 and active RAS physically interact. To determine whether the capacity of RAF-1 and MEK-1 to bind to RAS is dependent also on the activation state of the cells and to determine the extent to which these interactions can occur, binding assays were carried out with lysates of NIH 3T3 cells which had been either serum starved or stimulated with fetal bovine serum. RAF-1 association was observed exclusively with RAS:GMP-PNP, with no detectable RAF-1 associating with RAS:GDP (Fig. 1A, lanes 2 and 4 versus 1 and 3). This suggests that the identity of the guanine nucleotide loaded on RAS is the primary factor which influences RAF-1 binding. Analysis of the RAF-1 present in the supernatants from RAS beads produced the reciprocal result: no detectable RAF-1 was present in supernatants from RAS:GMP-PNP beads (Fig. 1B, lanes 2 and 4), while RAF-1 was present in supernatants from RAS:GDP beads (Fig. 1B, lanes 1 and 3), suggesting that all of the RAF-1 was able to bind RAS:GMP-PNP. Furthermore, all detectable RAF-1 was found in the cytosolic fraction, rather than the membrane fraction (data not shown). Since a substantial proportion of cellular RAS associates with membranes, these results suggest that the portion of RAF-1 which may associate with RAS in the cell is released at some point, perhaps as a result of RAS:GTP hydrolysis during the preparation of lysates, a process which requires approximately 1 h.

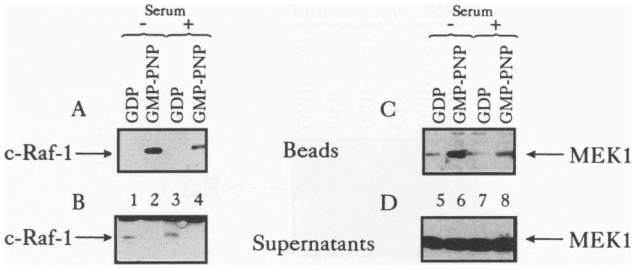


FIG. 1. RAF-1 and MEK-1 from cultured NIH 3T3 fibroblasts associate with immobilized RAS in a GMP-PNP-dependent manner, independently of cell stimulation. NIH 3T3 cells were serum starved for 16 h and stimulated with 20% fetal calf serum (lanes 3, 4, 7, and 8) or not stimulated (lanes 1, 2, 5, and 6) and lysed. Cytosolic fractions (100 μg) were incubated with 50 μg of immobilized RAS loaded with GDP or GMP-PNP, and precipitated or soluble RAF-1 (A and B) and MEK-1 proteins (C and D) were assayed by Western blot analysis and enhanced chemiluminescence. (A) Blot for RAF-1 from precipitated samples. (B) One-third of the total supernatant from panel A, blotted for RAF-1. (C) Proteins stripped from beads blotted for MEK-1. (D) One-third of the total supernatant was blotted for MEK-1.

In addition to RAF-1, MEK-1 was also found to be associated with the RAS complex in a GMP-PNP-dependent manner, irrespective of prior serum stimulation (Fig. 1C, lanes 6 and 8 versus 5 and 7). An estimated 1 to 5% of the total MEK-1 was associated with RAS:GMP-PNP (data not shown). In contrast with RAF-1, which bound exclusively to RAS:GMP-PNP, a small amount of MEK-1 was detected with RAS:GDP, suggesting a degree of nonspecific MEK-1 binding. This may be due to the excess quantity of MEK-1 present in the cell relative to RAF-1, since MEK-1 was not depleted from the lysates by binding to RAS beads in these experiments, with large amounts of MEK-1 remaining in the supernatants (Fig. 1D, lanes 5 to 8). Similar amounts of MEK-1, but not RAF-1, associated with unconjugated silica as with RAS:GDP silica, although far more cellular proteins associated nonspecifically with unconjugated silica than with RAS-coupled silica (data not shown).

**Association of RAF-1 and MEK-1 with immobilized RAS is sensitive to a peptide corresponding to the RAS effector domain.** Previous work with effector domain mutations of RAS and a peptide corresponding to this domain has demonstrated the importance of the effector domain in binding of RAF-1 to RAS. To determine whether the same region is necessary for binding of MEK-1 to RAS, we used as a competitor of this interaction an oligopeptide with the sequence YDPTIEDSYR KQVVID, corresponding to the effector region of c-Ha-RAS (residues 32 to 47) and common to all alleles of *ras*. A peptide

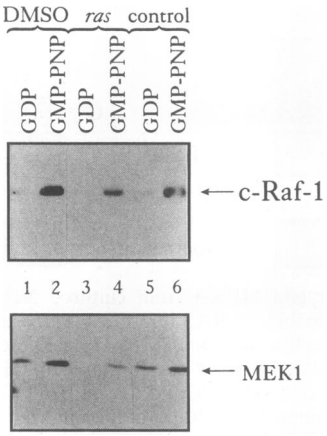


FIG. 2. Competition of RAF-1 and MEK-1 binding by RAS effector domain peptide. Cell lysate samples (100 μg) were incubated with 50 μg of immobilized RAS beads plus solvent alone (5% [vol/vol] DMSO) (lanes 1 and 2), RAS effector domain peptide (lanes 3 and 4), or an irrelevant peptide of similar hydrophobicity (lanes 5 and 6), at peptide concentrations of 1 mM. Proteins stripped off the beads were analyzed by Western blots probed against RAF-1 (upper panel) and MEK-1 (lower panel).

of the same sequence has previously been used to block signalling by RAS proteins (32). As shown in Fig. 2, incubation of lysates and beads with the RAS peptide resulted in a decrease of RAF-1 binding when compared with incubation with dimethyl sulfoxide (DMSO) solvent alone (lane 2 versus 4). In contrast, incubation with an irrelevant control peptide of similar size and hydrophobicity (KIGTAEPDYGALYEGR) did not detectably affect RAF-1 binding (Fig. 2, lane 2 versus 6). When the same blot was probed for MEK-1, the association of MEK-1 with immobilized RAS was likewise sensitive to the RAS effector domain peptide but not to DMSO alone or an irrelevant peptide (Fig. 2, lanes 2 and 6 versus 4). These results indicate that the same portion of the RAS molecule is required for association of both RAF-1 and MEK-1 to immobilized RAS. This dependence could occur by virtue of each of the molecules binding separately, to the same sequence of distinct RAS molecules, or if a ternary complex is formed, only one of the proteins interacting directly with the RAS effector domain.

**RAF-1 mediates MEK-1 interaction with immobilized RAS.** Data from expression studies with *Saccharomyces cerevisiae* suggest that RAF-1 associates directly with RAS and that MEK-1 might be able to associate with RAS if RAF-1 is simultaneously expressed (37). In addition, baculovirus expression of RAF-1 together with MEK-1 suggests that these proteins form a complex (15).

To determine whether MEK-1 from NIH 3T3 fibroblasts was associated with immobilized RAS through an intermediate protein such as RAF-1, we exploited our observation that incubation of cell lysates with immobilized RAS:GMP-PNP but not RAS:GDP resulted in the depletion of all detectable RAF-1 from the lysate, while MEK-1 was not depleted in either case (cf. Fig. 1B and D). Lysates were incubated with either RAS:GDP (Fig. 3A, lane 1) or RAS:GMP-PNP (Fig. 3A, lane 4), and the supernatants from these reaction mixtures were divided and incubated with fresh immobilized RAS beads loaded with either nucleotide (the GDP supernatant was divided between lanes 2 and 3, and the GMP-PNP supernatant was divided between lanes 5 and 6). While no detectable RAF-1 from fresh lysate associated with RAS:GDP (Fig. 3A, lane 1), the RAF-1 in this supernatant was able to associate with RAS:GMP-PNP but not with RAS:GDP (Fig. 3A, lane 3 versus 2). As previously mentioned, some degree of MEK-1 binding to RAS:GDP was seen in fresh lysate (Fig. 3A, lane 1). However, this quantity was very small compared with the large amount of MEK-1 from the RAS:GDP supernatant which associated with RAS:GMP-PNP (Fig. 3A, lane 3). These results indicate that the RAS:GDP incubation did not deplete the lysates of any component required for the association of RAF-1 or MEK-1 with the immobilized RAS complex.

In contrast, when fresh lysates were incubated with RAS:GMP-PNP, large amounts of RAF-1 and MEK-1 were found in association with immobilized RAS (Fig. 3A, lane 4). However, when these supernatants were subsequently probed with fresh RAS beads, no appreciable amount of RAF-1 or MEK-1 binding was detected (Fig. 3A, lanes 5 and 6). The supernatants of the serial binding assays were blotted for MEK-1, which was still in excess (Fig. 3B, lanes 2, 3, 5, and 6, corresponding to the appropriate lanes in Fig. 3A). These results indicate that while MEK-1 was not depleted by an incubation with RAS:GMP-PNP beads, RAF-1 was depleted, precluding all but residual levels of MEK-1 binding in the supernatant.

To determine whether the absence of RAF-1 (or an analogous protein) in these depleted lysates was limiting the ability of MEK-1 to associate with immobilized RAS, we reconstituted depleted lysates with partially pure baculovirus-expressed RAF-1. Whether supernatant was depleted with RAS:GDP beads (Fig. 3C, lanes 1 and 2) or RAS:GMP-PNP beads (Fig. 3C, lanes 3 and 4), the addition of exogenous RAF-1 facilitated equally high levels of GMP-PNP-dependent MEK-1 binding (Fig. 3C, lane 4 versus 2). The exogenous RAF-1 was free of contaminating MEK-1 (Fig. 3C, lane 5).

These results confirm that MEK-1 is unable to bind directly to immobilized RAS:GMP-PNP (even though it is present in excess) and that RAF-1 can mediate this interaction.

**Serum dependence of RAS:RAF-1 and RAS:MEK-1 interactions.** Association of RAF-1 with immobilized RAS occurred independently of prior stimulation of cells with serum (Fig. 1A, lane 2 versus 4). In all cases, RAF-1 from serum-stimulated lysates showed a mobility shift, indicating that RAF-1 was hyperphosphorylated (Fig. 1A, lane 4, and Fig. 1B, lane 3), suggesting that MAP kinase was active. Association of MEK-1 with RAS:GMP-PNP, like RAF-1, was independent of serum stimulation (Fig. 1C, lane 6 versus 8). These results suggest that complexes of RAS, RAF-1, and MEK-1 are able to form irrespective of the activation state of the cells and that the formation of these complexes in cells is dependent primarily on the production of RAS:GTP by mitogen stimulation.

**MEK-2 does not interact with immobilized RAS.** Two closely related MEK proteins are capable of activating MAP kinase, MEK-1 and MEK-2 (42). We attempted to detect MEK-2 in association with immobilized RAS and were unsuccessful, despite blotting with several different antisera. All antisera detected a band of the correct molecular weight in the supernatant (data not shown). Examination of the sequences of MEK-1 and MEK-2 revealed few regions of nonhomology, but one notable divergence encompassed T-292, a site of MEK-1 phosphorylation (4, 28). To assess the role of this site in the interaction of MEK-1 with immobilized RAS and to directly compare the interactions of MEK-1 and MEK-2 with the RAS signalling complex, we assayed lysates of cells overexpressing HA-tagged wild-type MEK-1, MEK-2, or a mutant MEK-1, T292A (a T-to-A change at position 292). The proteins are expressed to comparably high levels and can be enzymatically activated by serum stimulation (4). No signal was

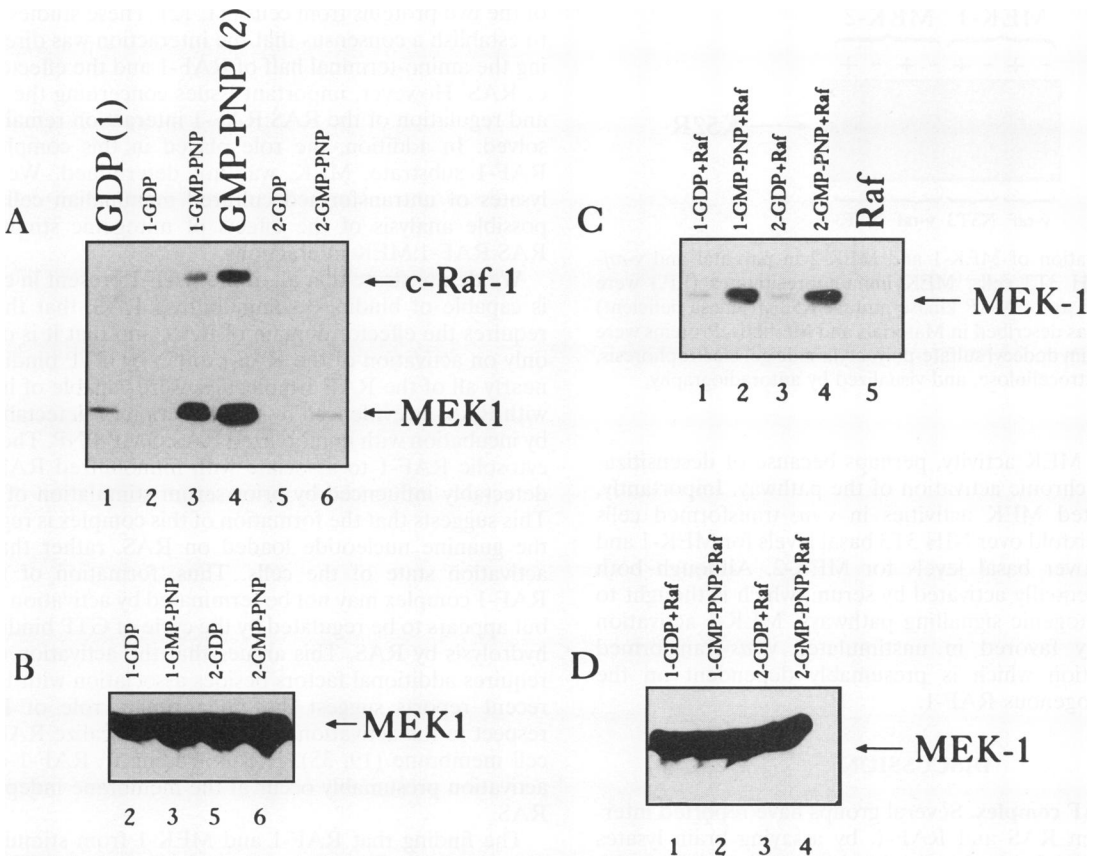


FIG. 3. Depletion of RAF-1 from cell lysates ablates MEK-1 binding. Fresh lysate (10 μg) was incubated with RAS:GDP (lane 1) or RAS:GMP-PNP (lane 4) for 30 min at 4°C. The supernatants were incubated with fresh beads (panel A: lanes 2 and 3 are from lane 1, and lanes 5 and 6 are from lane 4). (A) Blot probed for MEK-1 and RAF-1 sequentially, with both signals evident on the exposure shown. (B) Supernatants from panel A probed for MEK-1. (C) Depleted lysate prepared as for panel A and incubated with beads plus 1 μg of partially (5%) purified RAF-1. The blot was probed for MEK-1. (D) Supernatants from panel C probed for MEK-1.

detected from cells transfected with empty HA vector (Fig. 4A, lanes 1 and 2), but HA MEK-1 bound in a GMP-PNP-dependent manner (Fig. 4A, lanes 3 and 4). However, HA-tagged MEK-2 was not detected with RAS:GMP-PNP (Fig. 4A, lanes 5 and 6), and mutant MEK-1 (T292A) had a greatly reduced binding (Fig. 4A, lanes 7 and 8). All three proteins were present in approximately equal concentrations in the lysate (Fig. 4B). Prior serum stimulation had no effect on these binding assays (data not shown). These results suggest that

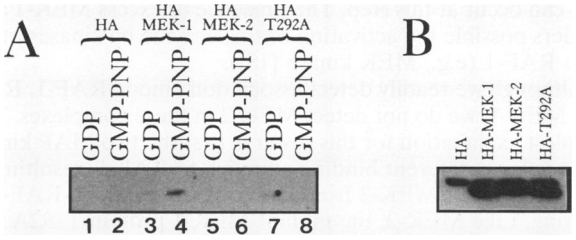


FIG. 4. MEK-2 and mutant MEK-1 (T292A) do not associate with immobilized RAS. Lysates were prepared and processed as described. (A) Blot of proteins stripped off of beads probed with anti-HA monoclonal antibody 12CA5; (B) lysate volume equal to that used in A probed with 12CA5. A nonspecific band running just above MEK is seen in all of the lanes.

MEK-1 is preferentially used in the RAS signalling complex and that T-292 of MEK-1 is important for its association with the RAS complex.

**Basal activity of MEK-1 and MEK-2 is equal in v-*raf*-transformed cells, but MEK-1 activity is elevated in v-*ras*-transformed cells.** To determine the physiological relevance of the above-described biochemical difference between MEK-1 and MEK-2, we assayed their basal and serum-stimulated activities in v-*raf*-transformed cells. It has been previously reported that v-RAF is able to activate both MEKs (42), and we observed no differences in the basal or platelet-derived growth factor (PDGF)-stimulated activities of MEK-2 versus MEK-1 in v-*raf*-transformed cells (Fig. 5). In several repeats of the experiment, inductions were between 5- and 10-fold for both MEKs, in both cell lines, irrespective of whether stimulations were with PDGF or serum. However, v-RAF is overexpressed in these cells and carries several structural alterations (including an extensive amino-terminal truncation) which could alter its substrate specificity relative to that of c-RAF-1.

To better analyze the activities of the two MEKs in the context of the RAS signalling pathway, we examined NIH 3T3 cells overexpressing c-RAS or transformed by v-*ras*. As summarized in Table 1, MEK activity was serum stimulated 20- to 30-fold in parental NIH 3T3 cells or in c-RAS overexpressing cells. v-*ras*-transformed cells were partially refractory to serum

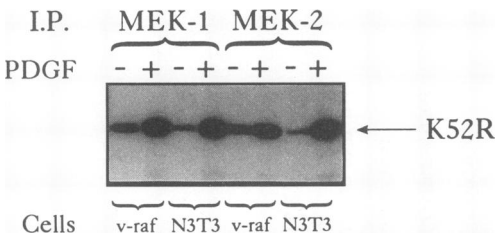


FIG. 5. Activation of MEK-1 and MEK-2 in parental and v-*raf*-transformed NIH 3T3 cells. MEK immunoprecipitates (I.P.) were incubated with 2 μg of MAP kinase mutant K52R (kinase deficient) and [γ-$^{32}$P]ATP as described in Materials and Methods. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized by autoradiography.

stimulation of MEK activity, perhaps because of desensitization following chronic activation of the pathway. Importantly, the unstimulated MEK activities in v-*ras*-transformed cells were induced sixfold over NIH 3T3 basal levels for MEK-1 and only twofold over basal levels for MEK-2. Although both proteins were equally activated by serum, which is thought to activate all mitogenic signalling pathways, MEK-1 activation was apparently favored in unstimulated v-*ras*-transformed cells, a condition which is presumably dependent on the activity of endogenous RAF-1.

## DISCUSSION

**The RAS:RAF complex.** Several groups have reported interactions between RAS and RAF-1, by assaying brain lysates with immobilized RAS (24), by in vitro binding assays with glutathione *S*-transferase fusion proteins, by the yeast two-hybrid coexpression system (37–39, 45), or by coprecipitation of the two proteins from cells (11, 12). These studies were able to establish a consensus that the interaction was direct, involving the amino-terminal half of RAF-1 and the effector domain of RAS. However, important issues concerning the specificity and regulation of the RAS:RAF-1 interaction remained unresolved. In addition, the role played in this complex by the RAF-1 substrate, MEK, was not determined. We analyzed lysates of untransformed cultured mammalian cells, making possible analysis of the effects of mitogenic stimulation on RAS:RAF-1:MEK interactions.

TABLE 1. Activities of MEK-1 and MEK-2[a]

| Cell line | IP | Stim | % Maximal activity (cpm) | Induction (fold) |
|---|---|---|---|---|
| NIH 3T3 (parental) | MEK-1 | − | 3.2 ± 0.5 | 1 |
| | MEK-2 | − | 4.1 ± 1.2 | 1 |
| | MEK-1 | + | 100 | 34.6 ± 6.1 |
| | MEK-2 | + | 100 | 21.8 ± 5.8 |
| c-RAS overexpressing | MEK-1 | − | 2.2 ± 0.5 | 0.7 ± 0.2 |
| | MEK-2 | − | 4.0 ± 2.3 | 0.6 ± 0.2 |
| | MEK-1 | + | 76.6 ± 5.9 | 28.0 ± 6.9 |
| | MEK-2 | + | 78.4 ± 7.1 | 17.6 ± 5.3 |
| v-*ras* transformed | MEK-1 | − | 17.8 ± 4.3 | 5.8 ± 1.2 |
| | MEK-2 | − | 9.8 ± 3.7 | 1.8 ± 0.5 |
| | MEK-1 | + | 37.0 ± 10.6 | 13.8 ± 3.8 |
| | MEK-2 | + | 42.8 ± 15.9 | 9.3 ± 1.6 |

[a] MEK immunoprecipitations and kinase assays were performed as described in Materials and Methods as well as in the legend to Fig. 5. Filters were stained, and MAP kinase K52R bands were excised and subjected to Cerenkov counting. For each condition, net counts per minute were calculated as observed counts minus preimmune serum control counts for that condition. Counts per minute were normalized to a percentage of maximal activity for each MEK in NIH 3T3 cells and then averaged over five repetitions of the experiment. Standard error values are shown. Fold induction is expressed relative to that for unstimulated NIH 3T3 cells. Stim, serum stimulation; IP, immunoprecipitate.

We demonstrate that all of the RAF-1 present in cell lysates is capable of binding to immobilized RAS, that the binding requires the effector domain of RAS, and that it is dependent only on activation of the RAS protein by GTP binding. All or nearly all of the RAF-1 molecules were capable of interacting with RAS, as evidenced by the depletion of detectable RAF-1 by incubation with immobilized RAS:GMP-PNP. The ability of cytosolic RAF-1 to associate with immobilized RAS was not detectably influenced by prior serum stimulation of the cells. This suggests that the formation of this complex is regulated by the guanine nucleotide loaded on RAS, rather than by the activation state of the cells. Thus, formation of the RAS:RAF-1 complex may not be terminated by activation of RAF-1 but appears to be regulated by the cycle of GTP binding to and hydrolysis by RAS. This argues that the activation of RAF-1 requires additional factors besides association with RAS. Two recent reports suggest that the primary role of RAS with respect to the activation of RAF-1 is to localize RAF-1 to the cell membrane (19, 35). Events leading to RAF-1 enzymatic activation presumably occur at the membrane independent of RAS.

The finding that RAF-1 and MEK-1 from stimulated cells can still bind to RAS raises the possibility that binding to RAS could direct RAF-1, or the RAF-1:MEK-1 complex, to specific substrates localized at cell membranes, and this localization could serve an additional, spatial regulatory role independent of the activation of MEK-1. For example, MAP kinase, which is activated by MEK-1, is able to phosphorylate threonine 669 in the juxtamembrane domain of the epidermal growth factor receptor (6). The MAP kinase molecules responsible for this phosphorylation could be locally activated by a RAS:RAF-1:MEK-1 complex in turn regulated by GRB-2/SOS (22), which itself would be bound to the receptor. The localization of the signalling complex to the plasma membrane could also facilitate MAP kinase-mediated cross talk with other signalling systems which function in the submembrane region.

**The RAF-1:MEK complex.** Depletion of all detectable RAF-1 from cell lysates by binding to immobilized RAS still leaves a majority of the MEK-1 protein in the supernatants. This suggests that MEK-1 is present in excess over RAF-1 in fibroblasts, consistent with the possibility that signal amplification can occur at this step. The presence of excess MEK-1 also renders possible the activation of this enzyme by kinases other than RAF-1 (e.g., MEK kinase [18]).

Although we readily detect association among RAF-1, RAS, and MEK-1, we do not detect MEK-2 in these complexes. The simplest explanation for this result is that the two MAP kinase kinases have different binding affinities to RAF-1, resulting in the exclusion of MEK-2 from the complex in which RAF-1 is limiting. Like MEK-2, the mutant MEK-1 protein T292A was also unable to bind to the RAS signalling complex, and since wild-type MEK-1 is phosphorylated on T-292, it is possible that this phosphorylation event controls the binding of MEK-1 to the RAS signalling complex. Interestingly, T-292 is located in the middle of a proline-rich loop common to MEK-1 and MEK-2, but MEK-2 lacks phosphorylatable residues in that

region. In other experiments, we have been able to immunoprecipitate RAF-1 with overexpressed MEK-1 and MEK-2; it is possible that specificity is imparted by the ternary complex.

In cells transformed by v-*ras*, in which the link between RAS and MEK is mediated by RAF-1, we found that MEK-1 had an elevated basal activity relative to that of MEK-2. This is consistent with the results of our in vitro binding experiments, which suggest that MEK-1 is physically linked to active RAS, while MEK-2 is not. It is also possible that differential desensitization accounts for this difference in activity. However, in v-*raf*-transformed cells, both MEKs are equally activated in the basal state. This is consistent with earlier results (42) and suggests that some redundancy in function exists between MEK-1 and MEK-2 or that v-RAF has a relaxed substrate specificity relative to that of RAF-1.

While our observed differences in basal MEK activities in v-*ras*-transformed cells are statistically valid, they are not striking. We suggest that this is consistent with a certain degree of functional redundancy between these related signalling molecules. Functional redundancy is also observed between members of the *src* family of tyrosine kinases, which can substitute for one another.

Activation of RAS is usually necessary for maximal activation of MAP kinases (9, 26, 36), suggesting that a component of the pathway to MEK-2 activation may be RAS dependent. While we suggest that MEK-1 is physically and functionally linked to the RAS signalling pathway and that some degree of MEK-2 activation can be achieved by a RAS-dependent pathway, optimal MEK-2 activation may require additional factors, some of which may be RAS independent.

The existence of two forms of MEK which can be differentially regulated provides the potential for flexible control over the MAP kinase signalling pathway. For example, MAP kinase is activated in two waves, a rapid acute phase (within 5 min) and a more prolonged phase (1 to 3 h) (23). In addition, a portion of the MAP kinase localizes to the nucleus following activation (5, 30, 34). The divergence in regulation between MEK-1 and MEK-2 could make possible the differential activation, timing, or localization of MAP kinases.

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